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| (51) International Patent Classification ⁶ : C12Q 1/68, C07H 21/04 | A1 | (11) International Publication Number: WO 99/46405 (43) International Publication Date: 16 September 1999 (16.09.99) |
| (21) International Application Number: PCT/US99/04251 (22) International Filing Date: 11 March 1999 (11.03.99) (30) Priority Data: 09/037,990 11 March 1998 (11.03.98) US (71) Applicant: E. & J. GALLO WINERY [US/US]; 600 Yosemite Boulevard, Modesto, CA 95354 (US). (72) Inventors: ENGEL, Stacia, R.; 4063-A Elmo Loop, Modesto, CA 95356 (US). DESCENZO, Richard, A.; 3808 Laurenburg Avenue, Modesto, CA 95357 (US). MORENZONI, Richard, A.; 2800 Warwick Lane, Modesto, CA 95350 (US). IRELAN, Nancy, A.; 2028 Monte Carlo Court, Modesto, CA 95350 (US). (74) Agents: SWECKER, Robert, S. et al.; Burns, Doane, Swecker & Mathis, L.L.P., P.O. Box 1404, Alexandria, VA 22313-1404 (US). | | (81) Designated States: AU, BR, CA, CN, JP, NZ, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| (54) Title: DETECTION OF FERMENTATION-RELATED MICROORGANISMS (57) Abstract Unique DNA sequences are provided which are useful in identifying different fermentation-related microorganisms, such as those involved in fermentations. These unique DNA sequences can be used to provide oligonucleotide primers in PCR based analysis for the identification of fermentation-related microorganisms. The DNA sequences of the present invention include the internal transcribed spacer (ITS) of the ribosomal RNA gene regions of particular fermentation-related microorganisms, as well as oligonucleotide primers which are derived from these regions which are capable of identifying the particular microorganism. | | |

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DETECTION OF FERMENTATION-RELATED MICROORGANISMS

Field of the Invention

The present invention relates to assays to detect fermentation-related microorganisms.

Description of the Related Art

In traditional wine-making, the indigenous yeasts ferment the grape must. Most modern wine-makers, however, inoculate with a pure culture of a selected yeast strain to ensure a rapid, reliable and predictable fermentation. It is thought that indigenous yeasts are suppressed by the competitive effect of addition of a high-density monoculture, but some evidence suggests that indigenous yeast can still participate in the fermentation. A range of commercial yeasts with different wine-making characteristics is available, and a number of those yeast strains may be used in a single winery. Furthermore, unwanted microorganisms may be present which lead to spoilage. Therefore, there is a need for a rapid, simple and accurate method for identifying microorganisms in starter cultures and fermentations.

Past techniques for detecting and identifying fermentation-related microorganisms, especially yeast, include colony morphology, fermentation performance, sugar fermentation tests, tolerance to various stresses (e.g., ethanol tolerance in brewing), phenotypes with functional relevance (e.g., flocculation in brewing), nutritional requirements (e.g., oxygen), and resistance and sensitivity levels of cycloheximide. These methods, however, have numerous disadvantages, including lengthy analysis periods, inability to differentiate, e.g., different strains of yeast, and lack of reproducibility.

Recent developments in molecular biology and protein chemistry have provided new methods for identifying microorganisms, including DNA restriction fragment length polymorphisms, protein electrophoresis patterns and chromosome fingerprinting. Such techniques have been used for identifying fermentation-related microorganisms. See, for example, Casey et al, *Journal of the American Society of Brewing Chemists*, 48(3):100-106, 1990; Degre et al, *American Journal of Enology and Viticulture*, 40(4):309-315, 1989; Guillaumon et al, *Systematic and Applied Microbiology*, 19:122-132, 1992; Hoeben et al, *Current Genetics*, 10:371-379, 1986; Mozina et al, *Letters in Applied Microbiology*, 24(4):311-315, 1997; Paffetti et al, *Research Microbiology*, 146:587-594, 1995; Panchal et al, *Journal of the Institute of Brewing*, 93:325-327, 1987; Querol et al, *Systematic and Applied Microbiology*, 15:439-446, 1992; Vezinhet et al, *Applied Microbiology and Biotechnology*, 32:568-571, 1990; and Vezinhet et al, *American Journal of Enology and Viticulture*, 43(1):83-86, 1992.

Polymerase chain reaction (PCR)-based techniques have also been used to detect fermentation-related microorganisms. See, for example, DeBarros Lopes et al, *Applied and Environmental Microbiology*, 62(12):4514-4520, 1996; Fell, *Molecular Marine Biology and Biotechnology*, 2(3):174-180, 1993; Fell, *Journal of Industrial Microbiology*, 14(6):475-477, 1995; Ibeas et al, *Applied and Environmental Microbiology*, 62(3):998-1003, 1996; Lavallee et al, *American Journal of Enology and Viticulture*, 45(1):86-91, 1994; Lieckfeldt et al, *Journal of Basic Microbiology*, 33(6):413-425, 1993; and Ness et al, *J. Sci. Food Agric.*, 62:89-94, 1993.

Ribosomal genes are suitable for use as molecular probe targets because of their high copy number. Non-transcribed and transcribed spacer sequences associated with ribosomal genes are usually poorly conserved and, thus, are

advantageously used as target sequences for the detection of recent evolutionary divergence. Fungal rRNA genes are organized in units. Each unit encodes mature subunits of 18S, 5.8S, and 28S rRNA. The internal transcribed spacer (ITS) region lies between the 18S and 28S rRNA genes and contains two
5 variable non-coding spacers (referred to as ITS1 and ITS2) and the 5.8S rRNA gene (White et al., 1990; In: *PCR Protocols*; Eds.: Innes et al.; pages 315-322). In addition, the transcriptional units are separated by non-transcribed spacer sequences (NTSs). The ITS and NTS sequences are particularly suitable for the detection of different fungal pathogens.

10 Kumeda et al (*Applied and Environmental Microbiology*, 62(8):2947-2952, 1996) describes use of PCR to amplify ribosomal DNA internal transcribed spacers in order to differentiate species of *Aspergillus* Section *Flavi*. The ITS1-5.8S-ITS2 region was amplified using universal primers, and the PCR product analyzed by the principle of single-strand conformation
15 polymorphism. In addition, Gardes et al (In: *Methods in Molecular Biology*, Vol. 50: *Species Diagnostics Protocols: PCR and Other Nucleic Acid Methods*, Ed. J.P. Clapp, Humana Press, Totowa, NJ, (1996) pp. 177-186) describes restriction fragment length polymorphism (RFLP) analysis of fungal ITS regions amplified by PCR.

20 The PCR amplification of fungal ITS has also been described using other than universal primers. These methods allow for more specificity in identifying classes of fungi, or particular species of fungi. Thus, Gardes and Bruns (*Molecular Ecology*, 2:113-118, 1993) identified ITS primers which allow differentiation of DNA from basidiomycetes against ascomycete DNA.
25 Identification of specific species has been observed using PCR primers directed to unique sequences in the ITS1 and/or ITS2 regions of fungal pathogens. See, for example, Hamelin et al, *Applied and Environmental Microbiology*, 62(11):4026-4031, 1996; Mazzola et al, *Phytopathology*, 86(4):354-360, 1996;

O’Gorman et al, *Canadian Journal of Botany*, 72:342-346, 1994; and U.S.

Patent No. 5,585,238 to Ligon et al.

The present invention addresses the problem of detecting and identifying fermentation-related microorganisms by PCR-based techniques.

5 **Summary of the Invention**

 The present invention is directed to the identification of different fermentation-related microorganisms, particularly those involved in the production of wine. The present invention provides DNA sequences which exhibit variability between different fermentation-related microorganisms. In particular, the present invention identifies regions of DNA sequence located in the internal transcribed spacer (ITS) of the ribosomal RNA gene regions of various fermentation-related microorganisms. Primers derived from the ITS can be used in polymerase chain reaction (PCR) based diagnostic assays to determine the presence or absence of specific fermentation-related microorganisms, including those involved in the production of wine. The primers can also be used as molecular probes to detect the presence of target DNA.

 Thus, in one aspect, the present invention provides an isolated double stranded nucleic acid of the full length ITS1 or ITS2 region of a fermentation-related microorganism. More particularly, the DNA sequence is selected from among Sequence ID NOS: 13 to 36.

 In another aspect, the present invention provides an oligonucleotide primer for identification of a fermentation-related microorganism, wherein the primer is a divergent portion of the ITS1 or ITS2 region of a fermentation-related microorganism. More particularly, the oligonucleotide primer is selected from among Sequence ID NOS: 65 to 98. Furthermore, the oligonucleotide primers may be selected from among sequences which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 65 to 98,

primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 65 to 98 contiguous with 1 to 15 nucleotide bases in the 5' and/or 3' direction of corresponding SEQ ID NOS: 37 to 64, and primers of 10 nucleotide bases or longer which contain at least 5 contiguous nucleotide bases from one of SEQ ID NOS: 65 to 98 contiguous with from 1 to 15 nucleotide bases in the 5' and/or 3' direction of corresponding SEQ ID NOS: 37 to 64. A pair of the foregoing oligonucleotide primers for use in the amplification-based detection of an ITS of a fermentation-related microorganism is also provided.

In yet another aspect, a method is provided for detection of a fermentation-related microorganism which comprises: (a) obtaining DNA from a fungal culture or colony isolated from a fermentation, or from an organism present in a fermentation beverage; (b) amplifying a part of the ITS of the fermentation-related microorganism using the DNA as a template in a polymerase chain reaction with the aforementioned oligonucleotide primers; and (c) visualizing the amplified part of the ITS sequence to determine whether the fermentation-related microorganism is present.

In still another aspect, kits are provided which are useful in detecting fermentation-related microorganisms.

Brief Description of the Sequences in the Sequence Listing:

- | | | |
|----|--------------|--|
| 20 | SEQ ID NO: 1 | DNA sequence for the internal transcribed spacer of <i>Saccharomyces cerevisiae</i> and <i>Saccharomyces bayanus</i> . |
| | SEQ ID NO: 2 | DNA sequence for the internal transcribed spacer of <i>Saccharomyces ludwigii</i> . |
| | SEQ ID NO: 3 | DNA sequence for the internal transcribed spacer of <i>Dekkera bruxellensis</i> . |
| 25 | SEQ ID NO: 4 | DNA sequence for the internal transcribed spacer of <i>Dekkera intermedia</i> . |

- SEQ ID NO: 5 DNA sequence for the internal transcribed spacer of *Botrytis cinerea*.
- SEQ ID NO: 6 DNA sequence for the internal transcribed spacer of *Penicillium crustosum*.
- 5 SEQ ID NO: 7 DNA sequence for the internal transcribed spacer of *Penicillium expansum*.
- SEQ ID NO: 8 DNA sequence for the internal transcribed spacer of *Hanseniaspora guilliermondii*.
- SEQ ID NO: 9 DNA sequence for the internal transcribed spacer of *Debaryomyces carsonii*.
- 10 SEQ ID NO: 10 DNA sequence for the internal transcribed spacer of *Pichia anomala*.
- SEQ ID NO: 11 DNA sequence for the internal transcribed spacer of *Pichia kluyveri*.
- 15 SEQ ID NO: 12 DNA sequence for the internal transcribed spacer of *Candida krusei*.
- SEQ ID NO: 13 DNA sequence for the ITS1 of *Saccharomyces cerevisiae* and *Saccharomyces bayanus*.
- SEQ ID NO: 14 DNA sequence for the ITS2 of *Saccharomyces cerevisiae* and *Saccharomyces bayanus*.
- 20 SEQ ID NO: 15 DNA sequence for the ITS1 of *Saccharomyces ludwigii*.
- SEQ ID NO: 16 DNA sequence for the ITS2 of *Saccharomyces ludwigii*.
- 25 SEQ ID NO: 17 DNA sequence for the ITS1 of *Dekkera bruxellensis*.
- SEQ ID NO: 18 DNA sequence for the ITS2 of *Dekkera bruxellensis*.
- SEQ ID NO: 19 DNA sequence for the ITS1 of *Dekkera intermedia*.
- SEQ ID NO: 20 DNA sequence for the ITS2 of *Dekkera intermedia*.
- SEQ ID NO: 21 DNA sequence for the ITS1 of *Botrytis cinerea*.

| | | |
|----|---------------|--|
| | SEQ ID NO: 22 | DNA sequence for the ITS2 of <i>Botrytis cinerea</i> . |
| | SEQ ID NO: 23 | DNA sequence for the ITS1 of <i>Penicillium crustosum</i> . |
| | SEQ ID NO: 24 | DNA sequence for the ITS2 of <i>Penicillium crustosum</i> . |
| | SEQ ID NO: 25 | DNA sequence for the ITS1 of <i>Penicillium expansum</i> . |
| 5 | SEQ ID NO: 26 | DNA sequence for the ITS2 of <i>Penicillium expansum</i> . |
| | SEQ ID NO: 27 | DNA sequence for the ITS1 of <i>Hanseniaspora guilliermondii</i> . |
| | SEQ ID NO: 28 | DNA sequence for the ITS2 of <i>Hanseniaspora guilliermondii</i> . |
| 10 | SEQ ID NO: 29 | DNA sequence for the ITS1 of <i>Debaryomyces carsonii</i> . |
| | SEQ ID NO: 30 | DNA sequence for the ITS2 of <i>Debaryomyces carsonii</i> . |
| | SEQ ID NO: 31 | DNA sequence for the ITS1 of <i>Pichia anomala</i> . |
| | SEQ ID NO: 32 | DNA sequence for the ITS2 of <i>Pichia anomala</i> . |
| | SEQ ID NO: 33 | DNA sequence for the ITS1 of <i>Pichia kluyveri</i> . |
| 15 | SEQ ID NO: 34 | DNA sequence for the ITS2 of <i>Pichia kluyveri</i> . |
| | SEQ ID NO: 35 | DNA sequence for the ITS1 of <i>Candida krusei</i> . |
| | SEQ ID NO: 36 | DNA sequence for the ITS2 of <i>Candida krusei</i> . |
| | SEQ ID NO: 37 | Oligonucleotide Sequence SXUITS1a. |
| | SEQ ID NO: 38 | Oligonucleotide Sequence SXLITS2a. |
| 20 | SEQ ID NO: 39 | Oligonucleotide Sequence SXUITS1b. |
| | SEQ ID NO: 40 | Oligonucleotide Sequence SXLITS2b. |
| | SEQ ID NO: 41 | Oligonucleotide Sequence SLUITS1a. |
| | SEQ ID NO: 42 | Oligonucleotide Sequence SLLITS2a. |
| | SEQ ID NO: 43 | Oligonucleotide Sequence SLUITS1b. |
| 25 | SEQ ID NO: 44 | Oligonucleotide Sequence SLLITS2b. |
| | SEQ ID NO: 45 | Oligonucleotide Sequence BRUITS1a. |
| | SEQ ID NO: 46 | Oligonucleotide Sequence BRLITS2. |
| | SEQ ID NO: 47 | Oligonucleotide Sequence BRUITS1b. |
| | SEQ ID NO: 48 | Oligonucleotide Sequence BCUITS1a. |

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| | SEQ ID NO: 49 | Oligonucleotide Sequence BCLITS2. |
| | SEQ ID NO: 50 | Oligonucleotide Sequence BCUITS1b. |
| | SEQ ID NO: 51 | Oligonucleotide Sequence PXUITS1a. |
| | SEQ ID NO: 52 | Oligonucleotide Sequence PXLITS2a. |
| 5 | SEQ ID NO: 53 | Oligonucleotide Sequence PXUITS1b. |
| | SEQ ID NO: 54 | Oligonucleotide Sequence PXLITS2b. |
| | SEQ ID NO: 55 | Oligonucleotide Sequence HGUITS1. |
| | SEQ ID NO: 56 | Oligonucleotide Sequence HGLITS2. |
| | SEQ ID NO: 57 | Oligonucleotide Sequence DXLITS2. |
| 10 | SEQ ID NO: 58 | Oligonucleotide Sequence PAUITS1. |
| | SEQ ID NO: 59 | Oligonucleotide Sequence PALITS2. |
| | SEQ ID NO: 60 | Oligonucleotide Sequence PKLITS2. |
| | SEQ ID NO: 61 | Oligonucleotide Sequence CKUITS1. |
| | SEQ ID NO: 62 | Oligonucleotide Sequence CKLITS2. |
| 15 | SEQ ID NO: 63 | Oligonucleotide Sequence SXU99. |
| | SEQ ID NO: 64 | Oligonucleotide Sequence SXL658. |
| | SEQ ID NO: 65 | Oligonucleotide Sequence SXU102. |
| | SEQ ID NO: 66 | Oligonucleotide Sequence SXL661. |
| | SEQ ID NO: 67 | Oligonucleotide Sequence SLU85. |
| 20 | SEQ ID NO: 68 | Oligonucleotide Sequence SLL635. |
| | SEQ ID NO: 69 | Oligonucleotide Sequence SLU88. |
| | SEQ ID NO: 70 | Oligonucleotide Sequence SLL636. |
| | SEQ ID NO: 71 | Oligonucleotide Sequence SLU136. |
| | SEQ ID NO: 72 | Oligonucleotide Sequence SLL634. |
| 25 | SEQ ID NO: 73 | Oligonucleotide Sequence BRU53A. |
| | SEQ ID NO: 74 | Oligonucleotide Sequence BRU53B. |
| | SEQ ID NO: 75 | Oligonucleotide Sequence BRU77. |
| | SEQ ID NO: 76 | Oligonucleotide Sequence BRL339. |
| | SEQ ID NO: 77 | Oligonucleotide Sequence BRL367. |

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| | SEQ ID NO: 78 | Oligonucleotide Sequence BRL390. |
| | SEQ ID NO: 79 | Oligonucleotide Sequence BCU136. |
| | SEQ ID NO: 80 | Oligonucleotide Sequence BCL393. |
| | SEQ ID NO: 81 | Oligonucleotide Sequence BCU142. |
| 5 | SEQ ID NO: 82 | Oligonucleotide Sequence PXU87. |
| | SEQ ID NO: 83 | Oligonucleotide Sequence PXL495. |
| | SEQ ID NO: 84 | Oligonucleotide Sequence PXU86. |
| | SEQ ID NO: 85 | Oligonucleotide Sequence PXL482. |
| | SEQ ID NO: 86 | Oligonucleotide Sequence PXL491. |
| 10 | SEQ ID NO: 87 | Oligonucleotide Sequence HGU193. |
| | SEQ ID NO: 88 | Oligonucleotide Sequence HGU231. |
| | SEQ ID NO: 89 | Oligonucleotide Sequence HGL601. |
| | SEQ ID NO: 90 | Oligonucleotide Sequence DXL447. |
| | SEQ ID NO: 91 | Oligonucleotide Sequence DXL526. |
| 15 | SEQ ID NO: 92 | Oligonucleotide Sequence PAU133. |
| | SEQ ID NO: 93 | Oligonucleotide Sequence PAL451. |
| | SEQ ID NO: 94 | Oligonucleotide Sequence PKL356. |
| | SEQ ID NO: 95 | Oligonucleotide Sequence CKU104. |
| | SEQ ID NO: 96 | Oligonucleotide Sequence CKL354. |
| 20 | SEQ ID NO: 97 | Oligonucleotide Sequence ITS5. |
| | SEQ ID NO: 98 | Oligonucleotide Sequence ITS4. |

Detailed Description Of The Invention

The present invention provides unique DNA sequences which are useful in identifying fermentation-related microorganisms. These unique DNA sequences can be used as primers in PCR-based analysis for the identification of fermentation-related microorganisms, or as molecular probes to detect the presence of DNA from fermentation-related microorganisms. The DNA sequences of the present invention include the internal transcribed spacer (ITS)

of the ribosomal RNA gene regions of specific fermentation-related microorganisms, as well as primers that are derived from these regions which are capable of identifying the particular microorganism.

The DNA sequences of the invention are from the ITS of the ribosomal RNA gene region of fermentation-related microorganisms. However, the present invention is not limited to detecting the presence of the microorganisms in fermentation operations, *i.e.*, the invention can be used to detect the presence of such microorganisms from any source. There is variability in the ITS DNA sequences from different microorganisms. The ITS sequences can be aligned and compared. Primers can be designed based on regions within the ITS regions that contain the greatest differences in sequence among the fermentation-related microorganisms. The sequences and primers based on these sequences can be used to identify specific microorganisms.

DNA sequences of particular interest include ITS DNA sequences from *Saccharomyces* sp., especially *Saccharomyces cerevisiae* and *Saccharomyces bayanus*; *Saccharomycodes* sp., especially *Saccharomycodes ludwigii*; *Dekkera* sp., especially *Dekkera bruxellensis* and *Dekkera intermedia*; *Botrytis* sp., especially *Botrytis cinerea*; *Penicillium* sp., especially *Penicillium crustosum* and *Penicillium expansum*; *Hanseniaspora* sp., especially *Hanseniaspora guilliermondii*; *Debaryomyces* sp., especially *Debaryomyces carsonii*; *Pichia* sp., especially *Pichia anomala* and *Pichia kluyveri*; and *Candida* sp., especially *Candida krusei*. The ITS DNA sequences, as well as primers of interest, are set forth in SEQUENCE ID NOS: 1-100. The sequences are useful in PCR-based identification of fermentation-related microorganisms.

Methods for use of the primer sequences of the invention in PCR analysis are well known in the art. For example, see U.S. Patent Nos. 4,683,195; 4,683,202 and 5,585,238, the contents of all of which are hereby incorporated by reference.

The primer sequences of the invention can also be used as molecular probes to detect the presence of target DNA. The T_m for the primers ranges from about 48-58° C at 50 mM salt. The hybridization temperature is approximately 5-10° C below the melting temperature. Thus, the primers are hybridized to target DNA typically at a temperature ranging from about 43-55° C. Final wash conditions generally range from about 45-55° C at about 36 mM salt concentration. Specific hybridization as used herein means the use of a final high stringency wash in about 0.2X SSPE (salt concentration of about 36 mM) at a temperature appropriate for the particular primer. 1X SSPE contains 10 mM NaH_2PO_4 , 180 mM NaCl, and 1 mM EDTA, at pH 7.4.

The ITS DNA sequences of the present invention can be cloned from fermentation-related microorganisms by methods known in the art. In general, the methods for the isolation of DNA from microorganism isolates are known. See, Raeder & Broda (1985) *Letters in Applied Microbiology* 2:17-20; Lee et al. (1990) *Fungal Genetics Newsletter* 35:23-24; and Lee and Taylor (1990) In: *PCR Protocols: A Guide to Methods and Applications*, Innes et al. (Eds.); pages 282-287; the contents of all of which are hereby incorporated by reference.

Alternatively, the ITS regions of interest can be identified by PCR amplification. Primers to amplify the entire ITS region can be synthesized according to White et al. (1990; In *PCR Protocols*; Eds.: Innes et al., pages 315-322, the contents of which are hereby incorporated by reference).

The ITS sequences were determined and the sequences were compared to locate divergences which might be useful to test in PCR to distinguish the different fermentation-related microorganisms. The sequences of the ITS regions which were determined are shown as Sequence ID NOS: 1 to 12. The DNA sequences for the ITS1 and ITS2 regions are shown as Sequence ID NOS: 13 to 36. From the identification of divergences, numerous primers were synthesized and tested in PCR-amplification. Purified microorganism

DNA and DNA isolated from fermentation cultures and colonies were used as templates for PCR-amplification. Thus, pairs of diagnostic primers were identified, *i.e.*, those which identified one particular fermentation-related microorganism species. Preferred primer combinations are able to distinguish
5 between the different microorganisms in, for example, fermentation cultures. Primer sequences are set forth in Sequence ID NOS: 65 to 98, with flanking sequences depicted in Sequence ID NOS: 37 to 64. Thus, while oligonucleotide primers selected from among Sequence ID NOS: 65 to 98 are preferred, primers may also be used which contain at least 10 contiguous
10 nucleotide bases from one of SEQ ID NOS: 65 to 98. Additionally, primers may be used which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 65 to 98 contiguous with 1 to 15 nucleotide bases in the 5' and/or 3' direction of corresponding SEQ ID NOS: 37 to 64, and primers of 10 nucleotide bases or longer which contain at least 5 contiguous nucleotide bases
15 from one of SEQ ID NOS: 65 to 98 contiguous with from 1 to 15 nucleotide bases in the 5' and/or 3' direction of corresponding SEQ ID NOS: 37 to 64.

The present invention provides numerous diagnostic primer combinations. The primers of the invention are designed based on sequence differences among the microorganism ITS regions. A minimum of one base
20 pair difference between sequences can permit design of a discriminatory primer. In general, primers should have a theoretical melting temperature between about 55 °C to about 65 °C to achieve good sensitivity, and should be void of significant secondary structure and 3' overlaps between primer combinations. Primers are generally at least about 10 nucleotide bases, more
25 preferably at least about 15 to about 20 nucleotide bases.

The oligonucleotide primers of the present invention are particularly useful in detecting microorganisms involved in fermentations, in particular, microorganisms selected from among *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomycodes ludwigii*, *Dekkera bruxellensis*,

Dekkera intermedia, *Botrytis cinerea*, *Penicillium crustosum*, *Penicillium expansum*, *Hanseniaspora guilliermondii*, *Debaryomyces carsonii*, *Pichia anomala*, *Pichia kluyveri*, and *Candida krusei*. However, the primers of the present invention can also be used to detect the presence of the foregoing
5 microorganisms from any source.

The present invention also relates to the preparation of "kits" containing elements for detecting fermentation-related microorganisms. Such a kit may comprise a carrier to receive therein one or more containers, such as tubes or vials. Unlabeled or detectably labeled oligonucleotide primers may be
10 contained in one or more of the containers. The oligonucleotide primers may be present in lyophilized form, or in an appropriate buffer. One or more enzymes or reagents for use in PCR reactions may be contained in one or more of the containers. The enzymes or reagents may be present alone or in admixture, and in lyophilized form or in appropriate buffers. The kit may also
15 contain any other component necessary for carrying out the present invention, such as buffers, extraction agents, enzymes, pipettes, plates, nucleic acids, nucleoside triphosphates, filter paper, gel materials, transfer materials, and autoradiography supplies.

The examples below illustrate typical experimental protocols which can
20 be used in the isolation of ITS sequences, the selection of suitable primer sequences, the testing of primers for selective and diagnostic efficacy, and the use of such primers to detect the presence of a fermentation-related microorganism. Such examples are provided by way of illustration and not by way of limitation.

Examples

Example 1

Culture of Yeast and Fungal Isolates

5 Viable isolates of *Botrytis cinerea*, *Penicillium crustosum*, and
 Penicillium expansum were obtained from the American Type Culture
 Collection (ATCC). Fungi were grown in 40 ml of Malt Yeast Extract Broth
 in 250 ml flasks inoculated with mycelial fragments from two-week-old
 cultures grown on Malt Yeast Extract Agar (MYEA). Liquid cultures were
 incubated at room temperature for 14 days without shaking. Malt Yeast
10 Extract Agar plates were inoculated with mycelia and grown for 2 weeks.
 Viable isolates of *Saccharomyces cerevisiae*, *Saccharomyces bayanus*,
 Saccharomycodes ludwigii, *Dekkera bruxellensis*, *Dekkera intermedia*, *Dekkera*
 anomala, *Hanseniaspora guilliermondii*, *Debaryomyces carsonii*, *Pichia*
 anomala, *Pichia kluyveri*, and *Candida krusei* were obtained from the ATCC or
15 from the Gallo Sonoma Winery (see Table 1). Yeasts were grown on any of
 several media of choice.

Example 2

Amplification and Sequencing of the Internal Transcribed Spacer (ITS) Regions

 The internal transcribed spacer region was amplified from the different
20 isolates directly from the fungal mycelium or the yeast colony using ITS5
 * (5'-GGAAGTAAAAGTCGTAACAAGG-3'; SEQ ID NO: 99) and ITS4
 (5'-TCCTCCGCTTATTGATATGC-3'; SEQ ID NO: 100). A sterile pipette
 tip was used to scrape a small amount of mycelia or colony off of the plate and
 deposited into a 250- μ l microcentrifuge tube containing 5 μ l each of GeneAmp[®]
25 10X PCR Buffer II and MgCl₂ solution (PE Applied Biosystems, Foster City,
 CA; part no. N808-0161), 0.2 mM each of dATP, dCTP, dGTP, and dTTP

(GeneAmp[®] dNTPs; PE Applied Biosystems, Foster City, CA; part no. N808-0007), approximately 25 pmole/ μ l each of ITS5 and ITS4, and 2.5 Units AmpliTaq[®] DNA polymerase (PE Applied Biosystems; part no. N808-0161). Reactions were run for 35 cycles of 30 s at 94 °C, 40 s at 58 °C, and 2 min at
5 72 °C, followed by a final elongation step at 72 °C for 10 min, on a Perkin Elmer GeneAmp[®] PCR System 9600 (PE Applied Biosystems). PCR products were purified using QIAquick[®] PCR Purification Kits (Qiagen Inc., Santa Clarita, CA) to remove any excess primers, nucleotides, and polymerases. Five microliters of the purified PCR products were run on a 1.2% agarose gel
10 with 5 μ l of pGEM⁻-3Zf(+) double-stranded DNA Control Template (0.2 g/L, PE Applied Biosystems) to approximate concentrations. All products were sequenced using the primers ITS5 and ITS4 (see sequences above; White et al., 1990; In: *PCR Protocols*; Eds.: Innes et al. pp. 315-322). Sequencing was performed on an PE Applied Biosystems 377 Automated DNA Sequencer[®]
15 using ABI PRISM[™] Dye Terminator Cycle Sequencing Ready Reaction Kits[®] (PE Applied Biosystems; part no. 402079). Cycle sequencing products were run over Centri-Sep[®] spin columns (Princeton Separations, Inc., Adelphia, NJ) to remove excess primers, dye-labeled terminators, nucleotides, and polymerases before being run on the automated sequencer.

20

Example 3

Selection of Species-Specific Primers

The ITS sequences of the *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomycodes ludwigii*, *Dekkera bruxellensis*, *Dekkera intermedia*, *Dekkera anomala*, *Botrytis cinerea*, *Penicillium crustosum*,
25 *Penicillium expansum*, *Hanseniaspora guilliermondii*, *Debaryomyces carsonii*, *Pichia anomala*, *Pichia kluyveri*, and *Candida krusei* isolates were aligned and

primers were designed using Oligo 5.0 (National Biosciences, Inc., Plymouth, MN) in regions of maximum sequence difference between the target species.

Example 4

Primer Synthesis

5 Primers were synthesized on an Applied Biosystems 394 DNA/RNA Synthesizer® using phosphoramidite chemistry.

Example 5

Verification of Primer Specificity to Target Species

10 Different annealing temperatures were tested to determine the optimal temperature for PCR for individual primers. In cases with multiple species-specific primers, different primer combinations were used to determine the best primer combination and annealing temperature to amplify a single species-specific DNA fragment. Species-specific amplification products were produced from primers designed from the ITS region between the 18S and 28S ribosomal
15 DNA subunits of each fungal strain of interest.

Example 6

Utilization of ITS sequences as diagnostic probes to hybridize with target DNA

1. Put chosen concentration of DNA sample in 100 ul of TE, pH 7.0.
2. Add 0.1 volume [10 µl] of 3.0 M NaOH, vortex to mix and incubate
20 at
65° C for 20 min to destroy the RNA and denature the DNA.
3. Spin down condensation. Allow samples to cool to room temp.
Neutralize by adding 1.0 volume [110 µl] of 2M ammonium acetate, pH 7.0,

vortex to mix. Spin down to remove solution off of cap. Refrigerate until slot blot apparatus is ready.

4. Apply to slot-blot apparatus according to manufacturers protocol; about 220 μ l to slot blot.

5 5. Label ITS sequence probe according to kit manufacturer's recommendation.

6. Prehybridize blots in 1.0% BSA; 1mM EDTA, 0.5 M NaHPO₄, pH 7.2, 7.0% sodium dodecyl sulfate for a minimum of 2 hr prior to adding the probe, and then hybridized for 16 hr at 45° C. Initial washes consist of two 30-
10 min washes in 1X SSPE/0.1% SDS at 50° C. Transfer blots to a plastic tray and wash in 1X SSPE for 1 hr, at 50° C with shaking. The final wash should consist of 15 min at 50° C in 0.2X SSPE.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that
15 various changes and modifications can be made therein without departing from the spirit and scope thereof.

Table 1
Sources of test isolates

| | Species name | ID number | Source |
|----|-------------------------------------|-----------|------------------------------|
| | <i>Saccharomyces cerevisiae</i> | GS061 | Gallo of Sonoma ¹ |
| 5 | <i>Saccharomyces cerevisiae</i> | GS117 | Gallo of Sonoma |
| | <i>Saccharomyces cerevisiae</i> | 4127 | ATCC ² |
| | <i>Saccharomyces bayanus</i> | 13056 | ATCC |
| | <i>Saccharomycodes ludwigii</i> | 34085 | ATCC |
| | <i>Saccharomycodes ludwigii</i> | 44299 | ATCC |
| 10 | <i>Dekkera bruxellensis</i> | Y153 | Gallo of Sonoma |
| | <i>Dekkera bruxellensis</i> | Y207 | Gallo of Sonoma |
| | <i>Dekkera bruxellensis</i> | 10560 | ATCC |
| | <i>Dekkera intermedia</i> | 34448 | ATCC |
| | <i>Dekkera anomala</i> | 10559 | ATCC |
| 15 | <i>Botrytis cinerea</i> | 20599 | ATCC |
| | <i>Penicillium crustosum</i> | 58616 | ATCC |
| | <i>Penicillium expansum</i> | 28885 | ATCC |
| | <i>Hanseniaspora guilliermondii</i> | GS014 | Gallo of Sonoma |
| | <i>Hanseniaspora guilliermondii</i> | GS057 | Gallo of Sonoma |
| 20 | <i>Hanseniaspora guilliermondii</i> | 66166 | ATCC |
| | <i>Debaryomyces carsonii</i> | Y443 | Gallo of Sonoma |
| | <i>Debaryomyces carsonii</i> | Y448 | Gallo of Sonoma |
| | <i>Debaryomyces carsonii</i> | 24214 | ATCC |
| | <i>Pichia anomala</i> | GS085 | Gallo of Sonoma |
| 25 | <i>Pichia anomala</i> | 34080 | ATCC |
| | <i>Pichia kluyveri</i> | GS070 | Gallo of Sonoma |
| | <i>Pichia kluyveri</i> | 64303 | ATCC |
| | <i>Candida krusei</i> | GS076 | Gallo of Sonoma |
| | <i>Candida krusei</i> | GS096 | Gallo of Sonoma |
| 30 | <i>Candida krusei</i> | GS108 | Gallo of Sonoma |

¹Gallo of Sonoma Winery, Healdsburg, CA, USA

²American Type Culture Collection, Rockville, MD, USA

Table 2

| | Target Organism | Primer Name | Primer Sequence |
|----|---------------------------------|-------------|--|
| | <i>Saccharomyces</i> sp. | SXU99 | 5'-CTTTTACTGGGCAAGAAGAC-3' (SEQ ID NO: 65) |
| | <i>Saccharomyces</i> sp. | SXL658 | 5'-AAGCACGCAGAGAAACC-3' (SEQ ID NO: 66) |
| 5 | <i>Saccharomyces</i> sp. | SXU102 | 5'-TTACTGGGCAAGAAGACAAG-3' (SEQ ID NO: 67) |
| | <i>Saccharomyces</i> sp. | SXL661 | 5'-CTCAAGCACGCAGAGAA-3' (SEQ ID NO: 68) |
| | <i>Saccharomycodes ludwigii</i> | SLU85 | 5'-GACTTTTCTTGGGGAGAG-3' (SEQ ID NO: 69) |
| 10 | <i>Saccharomycodes ludwigii</i> | SLL635 | 5'-TCACTAGTTGGGATAAACCT-3' (SEQ ID NO: 70) |
| | <i>Saccharomycodes ludwigii</i> | SLU88 | 5'-TTTTCTTGGGGAGAGG-3' (SEQ ID NO: 71) |
| | <i>Saccharomycodes ludwigii</i> | SLL636 | 5'-ATCACTAGTTGGGATAAACCT-3' (SEQ ID NO: 72) |
| 15 | <i>Saccharomycodes ludwigii</i> | SLU136 | 5'-CGGCTAGTAGTTGATGATT-3' (SEQ ID NO: 73) |
| | <i>Saccharomycodes ludwigii</i> | SLL634 | 5'-CACTAGTTGGGATAAACCTA-3' (SEQ ID NO: 74) |
| | <i>Dekkera</i> sp. | BRU53A | 5'-ATTACAGGATGCTGGGC-3' (SEQ ID NO: 75) |
| 20 | <i>Dekkera</i> sp. | BRU53B | 5'-ATTACAGGATGCTGGG-3' (SEQ ID NO: 76) |
| | <i>Dekkera</i> sp. | BRU77 | 5'-CGTGCAGACACGTGGAT-3' (SEQ ID NO: 77) |
| | <i>Dekkera</i> sp. | BRL339 | 5'-CTTTGAAGAAAACACCCTC-3' (SEQ ID NO: 78) |
| | <i>Dekkera</i> sp. | BRL367 | 5'-AATCATTATCCCCTCACTC-3' (SEQ ID NO: 79) |

| Target Organism | Primer Name | Primer Sequence |
|--|-------------|--|
| <i>Dekkera</i> sp. | BRL390 | 5'-TAATGAACGGCCGAAAC-3' (SEQ ID NO: 80) |
| <i>Botrytis cinerea</i> | BCU136 | 5'-TTGTATGCTCGCCAGAG-3' (SEQ ID NO: 81) |
| <i>Botrytis cinerea</i> | BCL393 | 5'-GCCTGCCATTACTGACA-3' (SEQ ID NO: 82) |
| <i>Botrytis cinerea</i> | BCU142 | 5'-GCTCGCCAGAGAATACC-3' (SEQ ID NO: 83) |
| 5 <i>Penicillium</i> sp. | PXU87 | 5'-ACCCGTCTTTATTTTACCTT-3' (SEQ ID NO: 84) |
| <i>Penicillium</i> sp. | PXL495 | 5'-CTACAGAGCGGGTGACA-3' (SEQ ID NO: 85) |
| <i>Penicillium</i> sp. | PXU86 | 5'-CACCCGTGTTTATTTTACCT-3' (SEQ ID NO: 86) |
| <i>Penicillium</i> sp. | PXL482 | 5'-CAAAGCCCCATACGC-3' (SEQ ID NO: 87) |
| <i>Penicillium</i> sp. | PXL491 | 5'-AGCGGGTGACAAAGC-3' (SEQ ID NO: 88) |
| 10 <i>Hanseniaspora guilliermondii</i> | HGU193 | 5'-CAACGTTTACACACATTGG-3' (SEQ ID NO: 89) |
| <i>Hanseniaspora guilliermondii</i> | HGU231 | 5'-AATTCTTTCTGCTTTGAATCG-3' (SEQ ID NO: 90) |
| 15 <i>Hanseniaspora guilliermondii</i> | HGL601 | 5'-CAGCGTCTCCAAAGAAGCTAA-3' (SEQ ID NO: 91) |
| <i>Debaryomyces carsonii</i> | DXL447 | 5'-GCAAACGCCTAGTTCGACTAA-3' (SEQ ID NO: 92) |
| <i>Debaryomyces carsonii</i> | DXL526 | 5'-ATTCAACGAGTTGGATAAACC-3' (SEQ ID NO: 93) |
| 20 <i>Pichia anomala</i> | PAU133 | 5'-GGCTTACTGCCCAAAGGTC-3' (SEQ ID NO: 94) |
| <i>Pichia anomala</i> | PAL451 | 5'-TGCTTATTAGTACACTCTTGC-3' (SEQ ID NO: 95) |

| Target Organism | Primer Name | Primer Sequence |
|------------------------|-------------|--|
| <i>Pichia kluyveri</i> | PKL356 | 5'-GTTTAGTTCACTTCGTCCACG-3' (SEQ ID NO: 96) |
| <i>Candida krusei</i> | CKU104 | 5'-CCTAAAATGTGGAATATAGCA-3' (SEQ ID NO: 97) |
| <i>Candida krusei</i> | CKL354 | 5'-ACGCTCTTTACACGTCGTC-3' (SEQ ID NO: 98) |

Table 3

ITS-derived Diagnostic PCR Primers

| | Target Organism | 5' Primer | 3' Primer | Approximate Size of PCR Product (bp) |
|----|---------------------------------|-----------|-----------|--------------------------------------|
| 5 | <i>Saccharomyces sp.</i> | SXU99 | ITS4 | 750 |
| | <i>Saccharomyces sp.</i> | SXU102 | ITS4 | 750 |
| | <i>Saccharomyces sp.</i> | ITS5 | SXL658 | 695 |
| | <i>Saccharomyces sp.</i> | ITS5 | SXL661 | 695 |
| | <i>Saccharomyces sp.</i> | SXU99 | SXL658 | 560 |
| 10 | <i>Saccharomyces sp.</i> | SXU99 | SXL661 | 560 |
| | <i>Saccharomyces sp.</i> | SXU102 | SXL658 | 560 |
| | <i>Saccharomyces sp.</i> | SXU102 | SXL661 | 560 |
| | <i>Saccharomycodes ludwigii</i> | SLU85 | ITS4 | 660 |
| | <i>Saccharomycodes ludwigii</i> | SLU88 | ITS4 | 660 |
| 15 | <i>Saccharomycodes ludwigii</i> | SLU136 | ITS4 | 610 |
| | <i>Saccharomycodes ludwigii</i> | ITS5 | SLL635 | 670 |
| | <i>Saccharomycodes ludwigii</i> | ITS5 | SLL636 | 670 |
| | <i>Saccharomycodes ludwigii</i> | ITS5 | SLL634 | 670 |
| | <i>Saccharomycodes ludwigii</i> | SLU85 | SLL635 | 565 |
| 20 | <i>Saccharomycodes ludwigii</i> | SLU85 | SLL636 | 565 |
| | <i>Saccharomycodes ludwigii</i> | SLU85 | SLL634 | 565 |
| | <i>Saccharomycodes ludwigii</i> | SLU88 | SLL635 | 565 |
| | <i>Saccharomycodes ludwigii</i> | SLU88 | SLL636 | 565 |
| | <i>Saccharomycodes ludwigii</i> | SLU88 | SLL634 | 565 |
| 25 | <i>Saccharomycodes ludwigii</i> | SLU136 | SLL635 | 500 |
| | <i>Saccharomycodes ludwigii</i> | SLU136 | SLL636 | 500 |
| | <i>Saccharomycodes ludwigii</i> | SLU136 | SLL634 | 500 |
| | <i>Dekkera sp.</i> | BRU53A | ITS4 | 450 |
| | <i>Dekkera sp.</i> | BRU53B | ITS4 | 450 |
| 30 | <i>Dekkera sp.</i> | BRU77 | ITS4 | 450 |
| | <i>Dekkera sp.</i> | ITS5 | BRL339 | 375 |
| | <i>Dekkera bruxellensis</i> | ITS5 | BRL367 | 400 |
| | <i>Dekkera sp.</i> | ITS5 | BRL390 | 425 |
| | <i>Dekkera sp.</i> | BRU53A | BRL339 | 290 |
| 35 | <i>Dekkera bruxellensis</i> | BRU53A | BRL367 | 320 |
| | <i>Dekkera sp.</i> | BRU53A | BRL390 | 340 |

| | | | | |
|----|-------------------------------------|--------|--------|-----|
| | <i>Dekkera sp.</i> | BRU53B | BRL339 | 290 |
| | <i>Dekkera bruxellensis</i> | BRU53B | BRL367 | 320 |
| | <i>Dekkera sp.</i> | BRU53B | BRL390 | 340 |
| | <i>Dekkera sp.</i> | BRU77 | BRL339 | 260 |
| 5 | <i>Dekkera bruxellensis</i> | BRU77 | BRL367 | 290 |
| | <i>Dekkera sp.</i> | BRU77 | BRL390 | 310 |
| | <i>Botrytis cinerea</i> | BCU136 | ITS4 | 350 |
| | <i>Botrytis cinerea</i> | BCU142 | ITS4 | 345 |
| | <i>Botrytis cinerea</i> | ITS5 | BCL393 | 420 |
| 10 | <i>Botrytis cinerea</i> | BCU136 | BCL393 | 270 |
| | <i>Botrytis cinerea</i> | BCU142 | BCL393 | 265 |
| | <i>Penicillium sp.</i> | PXU87 | ITS4 | 410 |
| | <i>Penicillium sp.</i> | PXU86 | ITS4 | 410 |
| | <i>Penicillium sp.</i> | ITS5 | PXL495 | 530 |
| 15 | <i>Penicillium sp.</i> | ITS5 | PXL482 | 520 |
| | <i>Penicillium sp.</i> | ITS5 | PXL491 | 530 |
| | <i>Penicillium sp.</i> | PXU87 | PXL495 | 405 |
| | <i>Penicillium sp.</i> | PXU87 | PXL482 | 395 |
| | <i>Penicillium sp.</i> | PXU87 | PXL491 | 405 |
| 20 | <i>Hanseniaspora guilliermondii</i> | HGU193 | ITS4 | 530 |
| | <i>Hanseniaspora guilliermondii</i> | HGU231 | ITS4 | 490 |
| | <i>Hanseniaspora guilliermondii</i> | ITS5 | HGL601 | 630 |
| 25 | <i>Hanseniaspora guilliermondii</i> | HGU193 | HGL601 | 420 |
| | <i>Hanseniaspora guilliermondii</i> | HGU231 | HGL601 | 380 |
| 30 | <i>Debaryomyces carsonii</i> | ITS5 | DXL447 | 480 |
| | <i>Debaryomyces carsonii</i> | ITS5 | DXL526 | 560 |
| | <i>Pichia anomala</i> | PAU133 | ITS4 | 505 |
| | <i>Pichia anomala</i> | ITS5 | PAL451 | 480 |
| | <i>Pichia anomala</i> | PAU133 | PAL451 | 320 |
| 35 | <i>Pichia kluyveri</i> | ITS5 | PKL356 | 390 |
| | <i>Candida krusei</i> | CKU104 | ITS4 | 440 |
| | <i>Candida krusei</i> | ITS5 | CKL354 | 385 |

We claim:

1. An isolated double stranded nucleic acid selected from the group consisting of SEQ ID NOS: 13 to 36.

2. An isolated nucleic acid which specifically hybridizes with the
5 nucleic acid of claim 1.

3. An oligonucleotide sequence for identification of a fermentation-related microorganism, wherein said sequence is selected from the group consisting of SEQ ID NOS: 37 to 64.

4. An oligonucleotide primer which is a fragment of the sequences
10 according to claim 3, and which specifically hybridizes to the ITS1 or ITS2 of *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomycodes ludwigii*, *Dekkera bruxellensis*, *Dekkera intermedia*, *Botrytis cinerea*, *Penicillium crustosum*, *Penicillium expansum*, *Hanseniaspora guilliermondii*, *Debaryomyces carsonii*, *Pichia anomala*, *Pichia kluyveri* or *Candida krusei*.

15 5. An oligonucleotide primer for identification of a fermentation-related microorganism, wherein said primer is selected from the group consisting of primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 65 to 98, primers which contain at least 10 contiguous nucleotide
20 bases from one of SEQ ID NOS: 65 to 98 contiguous with 1 to 15 nucleotide bases in the 5' and/or 3' direction of corresponding SEQ ID NOS: 37 to 64, and primers of 10 nucleotide bases or longer which contain at least 5 contiguous nucleotide bases from one of SEQ ID NOS: 65 to 98 contiguous with from 1 to 15 nucleotide bases in the 5' and/or 3' direction of corresponding SEQ ID NOS: 37 to 64.

6. A pair of oligonucleotide primers for use in the amplification-based detection of an internal transcribed spacer sequence of a fermentation-related microorganism, wherein the primers are selected from the group consisting of primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 65 to 98, primers which contain at least 10 contiguous nucleotide bases from one of SEQ ID NOS: 65 to 98 contiguous with 1 to 15 nucleotide bases in the 5' and/or 3' direction of corresponding SEQ ID NOS: 37 to 64, and primers of 10 bases or longer which contain at least 5 contiguous nucleotide bases from one of SEQ ID NOS: 65 to 98 contiguous with from 1 to 15 nucleotide bases in the 5' and/or 3' direction of corresponding SEQ ID NOS: 37 to 64.

7. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 65 and SEQ ID NO: 66.

8. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 67 and SEQ ID NO: 68.

9. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 69 and SEQ ID NO: 70.

10. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 71 and SEQ ID NO: 72.

11. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 73 and SEQ ID NO: 74.

12. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 75 and SEQ ID NO: 78.

13. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 76 and SEQ ID NO: 79.

14. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 77 and SEQ ID NO: 80.

5 15. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 81 and SEQ ID NO: 82.

16. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 85 and SEQ ID NO: 83.

10 17. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 84 and SEQ ID NO: 85.

18. The pair oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 86 and SEQ ID NO: 87.

19. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 86 and SEQ ID NO: 88.

15 20. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 89 and SEQ ID NO: 91.

21. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 90 and SEQ ID NO: 91.

20 22. The pair oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 94 and SEQ ID NO: 95.

23. The pair of oligonucleotide primers according to claim 6 which comprises SEQ ID NO: 97 and SEQ ID NO: 98.

24. A method for detection of a fermentation-related microorganism comprising:

- 5 (a) obtaining DNA from a fungal culture or colony isolated from a fermentation, or from an organism present in a fermentation beverage;
- (b) amplifying a part of the internal transcribed spacer sequence of said fermentation-related microorganism using said DNA as a
- 10 template in a polymerase chain reaction with a pair of oligonucleotide primers according to claim 6; and
- (c) visualizing said amplified part of the internal transcribed spacer sequence to determine whether said fermentation-related microorganism is present.

15 25. The method according to claim 24, wherein said fermentation-related microorganism is selected from the group consisting of *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomycodes ludwigii*, *Dekkera bruxellensis*, *Dekkera intermedia*, *Botrytis cinerea*, *Penicillium crustosum*, *Penicillium expansum*, *Hanseniaspora guilliermondii*, *Debaryomyces carsonii*,

20 *Pichia anomala*, *Pichia kluyveri*, and *Candida krusei*.

26. The method according to claim 24, wherein said fermentation culture or fermentation beverage is a wine fermentation culture or wine fermentation beverage.

27. The method according to claim 24, wherein the pair of

25 oligonucleotide primers comprises SEQ ID NO: 65 and SEQ ID NO: 66.

28. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 67 and SEQ ID NO: 68.

29. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 69 and SEQ ID NO: 70.

5 30. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 71 and SEQ ID NO: 72.

31. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 73 and SEQ ID NO: 74.

10 32. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 75 and SEQ ID NO: 78.

33. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 76 and SEQ ID NO: 79.

34. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 77 and SEQ ID NO: 80.

15 35. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 81 and SEQ ID NO: 82.

36. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 82 and SEQ ID NO: 83.

20 37. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 84 and SEQ ID NO: 85.

38. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 86 and SEQ ID NO: 87.

39. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 86 and SEQ ID NO: 88.

5 40. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 89 and SEQ ID NO: 91.

41. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 90 and SEQ ID NO: 91.

10 42. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 94 and SEQ ID NO: 95.

43. The method according to claim 24, wherein the pair of oligonucleotide primers comprises SEQ ID NO: 97 and SEQ ID NO: 98.

15 44. A kit comprising a carrier to receive therein one or more containers, at least one of said containers comprising an oligonucleotide primer according to claim 5.

45. A kit comprising a carrier to receive therein one or more containers, at least one of said containers comprising a pair of oligonucleotide primers according to claim 6.

Sequence Listing

SEQ ID NO: 1 DNA sequence for the internal transcribed spacer of
Saccharomyces cerevisiae and *Saccharomyces bayanus*.

5' AAGAAATTTAATAATTTTGAAAATGGATTTTTTTGTTTT
5 GGCAAGAGCATGAGAGCTTTTACTGGGCAAGAAGACAA
GAGATGGAGAGTCCACCCGGGCCTGCGCTTAAGTGCGC
GGTCTTGCTAGGCTTGTAAGTTTCTTTCTTGCTATTCCA
AACGGTGAGAGATTTCTGTGCTTTTGTTATAGGACAATT
AAAACCGTTTCAATACAACACACTGTGGAGTTTTCATAT
10 CTTTGCAACTTTTTCTTTGGGCATTCGAGCAATCGGGGC
CCAGAGGTAACAAACACAAACAATTTTATCTATTCATTA
AATTTTGTCAAAAACAAGAATTTTCGTAACCTGGAAATT
TTAAAATATTAAAACTTTCAACAACGGATCTCTTGGTT
CTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTA
15 ATGTGAATTGCAGAATCCGTGAATCATCGAATCTTTGA
ACGCACATTGCGCCCTTGGTATTCCAGGGGGCATGCCT
GTTTGAGCGTCATTTCTTCTCAAACATTCTGTTTGGTA
GTGAGTGATACTCTTTGGAGTAACTTGAAATTGCTGGC
CTTTTCATTGGATGTTTTTTTTTCCAAAGAGAGGTTTCTC
20 TCGGTGCTTGAGGTATAATGCAAGTACGGTCGTTTTAGG
TTTTACCAACTGCGGCTAATCTTTTTTTATACTGAGCGT
ATTGGAACGTTATCGATAAGAAGAGAGCGTCTAGGCGA
ACAATGTTCTTAAAGT 3'

SEQ ID NO: 2 DNA sequence for the internal transcribed spacer of
Saccharomyces ludwigii.

5' AAGAAAAAACTGTTTATAAACAGACGGTAGACTTTTC
TTGGGGAGAGGTTGTTGATTGCTTTGGCCTGCGCTTAAC
TGCGCGGCTAGTAGTTGATGATTTTGTTATTATCCGAGA
CGAAGGAAACGTCTGATTTTAAAAACATTATACACTTTG
30 GAGTACTTTTTTAAATGTATTTCTTCCTTGGACGAGCAA
TTGTTCAAGGGTCAATAAACACAAACAATTTTTTTTTTA
TTTAAATTTAAATAATTCAAAATATATCATTCTTTTA
TTAGGAATATAAAAAATTTTAAACCTTTCAACAACGGATC
TCTTGGTTCTCGCATCGATGAAGAACGTAGCGAATTGCG
35 ATAAGTAATGTGAATTGCAGATTTTCGTGAATCATTGAA
TTTTTGAACGCACATTGCGCCCTTGGTATTCCAAAGGG
CATGCCTGTTTGAGCGTCATTTCTTCTCAAAGAGTTT
TTTTATTCTTTGGTTGTGAGTGATACTCTTCTTTTAC

AGGGAAGGGGTAACTTGAAATTGTTGCCTAGCAAAGA
AGAATTTTGATTGAAATTTCTTGTTTATTACTATTAGGT
TTATCCCAACTAGTGATTATTGAGAGTTTTTATTACAGA
GTCTTTTCACTTGCTATAATACTATTCTATAAGT 3'

5 SEQ ID NO: 3 DNA sequence for the internal transcribed spacer of
Dekkera bruxellensis.

5' CAGGATGCTGGGCGCAAGCCCGTGCAGACACGTGGATA
AGCAAGGATAAAAATACATTAAATTTATTTAGTTTAGTC
AAGAAAGAATTTTAAACTTTCAACAATGGATCTCTTG
10 TTCTCGCGTCGATGAAGAGCGCAGCGGAATTGCGATAC
TTAATGTGAATTGCAGATTTTCGTGAATCATCGAGTTCT
TGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCAT
GCCTGTTTGAGCGTCATTTCCCTTCTCACTATTTAGTGGT
TATGAGATTACACGAGGGTGTTTTCTTCAAAGGAAAGA
15 GGGGAGAGTGAGGGGATAATGATTAAAGTTTCGGCCG
TTCATTATTTTTTCTTCTCCCCCAGTTATCAAGT 3'

SEQ ID NO: 4 DNA sequence for the internal transcribed spacer of
Dekkera intermedia.

5' CAGGATGCTGGGCGCAAGCCCGTGCAGACACGTGGATA
AGTAAGGATAAAAATACATTAAATTTATTTAGTTTATAGT
20 CAAGAAAGAATTTTAAACTTTCAACAATGGATCTCTTG
GTTCTCGCGTCGATGAAGAGCGCAGCGAATTGCGATAC
TTAATGTGAATTGCAGATTTTCGTGAATCATCGAGTTCT
TGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCAT
25 GCCTGTTTGAGCGTCATTTCCCTTCTCACTATTTAGTGGT
TATGAGATTACACGAGGGTGTTTTCTTCAAAGGAAAGA
GGGGAGAGTGAGGGGATAATGATTAAAGTTTCGGCCG
TTCATTATTTTTTCTTCTCCCCCAGTTATCAAGT 3'

30 SEQ ID NO: 5 DNA sequence for the internal transcribed spacer of
Botrytis cinerea.

5' CAGAGTTCATGCCCCGAAAGGGTAGACCTCCCACCTTG
TGTATTATTACTTTGTTGCTTTGGCGAGCTGCTTTCGGG
CCTTGTATGCTCGCCAGAGAATACCAAACTCTTTTTAT
TAATGTCGTCTGAGTACTATATAATAGTTAAACTTTCA

5 ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
TGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGG
TATTCGGGGGGGCATGCCTGTTTCGAGCGTCATTTCAACC
CTCAAGCTTAGCTTGGTATTGAGTCTATGTCAGTAATGG
CAGGCTCTAAAATCAGTGGCGGCGCCGCTGGGTCCTGA
ACGTAGTAATATCTCTCGTTACAGGTTCTCGGTGTGCTT
CTGCCAAAACCCAAATTTTCTATGG 3'

10 SEQ ID NO: 6 DNA sequence for the internal transcribed spacer of
Penicillium crustosum.

5' CCGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTG
TTATTTTACCTTGTTGCTTCGGCGGGCCCGCCTTAAC
GGCCGCCGGGGGGCTTACGCCCCGGGCCCCGCGCCCGC
CGAAGACACCCTCGAACTCTGTCTGAAGATTGAAGTCT
15 GAGTGAAAATATAAATTATTTAAACCTTCAACAACGG
ATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAA
TGCGATACGTAATGTGAATTGCAAATTCAGTGAATCATC
GAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGG
GGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCC
20 GGCTTGTGTGTTGGGCCCCGTCCCCCGATCTCCGGGGGA
CGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCT
CGAGCGTATGGGGCTTTGTACCCGCTCTGTAGGCCCGG
CCGGCGCTTGCCGATCAACCCAAATTTTATCCAGG 3'

25 SEQ ID NO: 7 DNA sequence for the internal transcribed spacer of
Penicillium expansum.

5' CCGAGTGAGGGCCCTTTGGGTCCAACCTCCCACCCGTG
TTATTTACCTCGTTGCTTCGGCGGGCCCGCCTTAAC
CCGCCGGGGGGCTCACGCCCCGGGCCCCGCGCCCGCCG
AAGACACCCCGAACTCTGCCTGAAGATTGTCGTCTGA
30 GTGAAAATATAAATTATTTAAACCTTCAACAACGGATC
TCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGC
GATACGTAATGTGAATTGCAAATTCAGTGAATCATCGA
GTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGG
GCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGG
35 CTTGTGTGTTGGGCCCCGTCTCCGATTCCGGGGGACGG
GCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGA
GCGTATGGGGCTTTGTACCCGCTCTGTAGGCCCGGCCG
CGCTTGCCGATCAACCCAAATTTTATCCAGGTTGACC

TCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATAT
CAATAAGCGGAGGAAA 3'

SEQ ID NO: 8 DNA sequence for the internal transcribed spacer of
Hanseniaspora guilliermondii.

5 5' GATTGAATTATCATTGTTGCTCGAGTTCTAGTTTTAGAT
CTTTTACAATAATGTGTATCTTTATTGAAGATGTGCGCT
TAATTGCGCTGCTTTTTTAAAGTGTGCGCAGTAGAAGTAA
TCTTGCTTGAATCTCAGTCAACGTTTACACACATTGGAG
TTTTTTTACTTTAATTTAATTCCTTCTGCTTTGAATCGAA
10 AGGTTCAAGGCAAAAAACAAACACAAACAATTTTATTT
TATTATAATTTTTTAAACTAAACCAAAATTCCTAACGGA
AATTTTAAAATAATTTAAAACTTTCAACAACGGATCTCT
TGGTTCTCGCATCGATGAAGAACGTAGCGAATTGCGAT
AAKTAATGTGAATTGCAGATACTCGTGAATCATTGAATT
15 TTTGAACGCACATTGCGCCCTTGAGCATTCTCAAGGGCA
TGCCTGTTTGAGCGTCATTCCTTCTCAAAAGATAATTT
TTTATTTTTTGGTTGTGGGCGATACTCAGGGTTAGCTTG
AAATTGAAGATTGTTTCAATCTTTTTTAATTCAACACTT
AGCTTCTTTGGAGACGCTGTTCTCGCTGTGATGTATTTA
20 TGAATTTATTCGTTTTACTTTACAAGGGAAATGGTAATG
TACCTTAGGCAAAGGGTTGCTTTTAATATTCATCAAGT
3'

SEQ ID NO: 9 DNA sequence for the internal transcribed spacer of
Debaryomyces carsonii.

25 5' CAGTATTCTTTTTGCCAGCGCTTAACTGCGCGGCGAAAT
AAACCTTACACACAATGTTTTTTGTTATTACAGGAACTT
TTGCTTTGGCTTGTCTCTAGAAATAGAGTTGGGCCAAAG
GTTTAAACTAACTTCAATTTATTTGAACTATTTTTCTTAT
TGAAATGTCACTTTGTGATTAAATTCAAAAAATCTTCA
30 AAACCTTCAACAACGGATCTCTTGGTTCTCGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATATGAATTGCA
GATTTTCGTGAATCATCGAATCTTTGAACGCACATTGCG
CCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCA
TTTCTCTCTCAAACCTTAGGGTTTGGTATTGAGTGATAC
35 TCTTAGTCGAACTAGGCGTTTGCTTGAAATGTATCGGCA
TGAGTGGTACTAGATTAGTGCTTCAGATTTTTCAATGTA
TTAGGTTTATCCAACCTCGTTGAATAGTCTGATGGCAAAGT

GTTTAGTAACTATGGCTCGGCCTAACAACAACAAACAA
GT 3'

SEQ ID NO: 10 DNA sequence for the internal transcribed spacer of
Pichia anomala.

5 5' TAGTATTCTATTGCCAGCGCTTAATTGCGCGGCGATAAA
CCTTACACACATTGTCTAGTTTTTTTGAACTTTGCTTTGG
GTGGTGAGCCTGGCTTACTGCCCAAAGGTCTAAACACA
TTTTTTTAATGTTAAAACCTTTAACCAATAGTCATGAAA
ATTTTTTAACAAAAATTTAAATCTTCAAACTTTCAACAA
10 CGGATCTCTTGTTCTCGCAACGATGAAGAACGCAGCG
AAATGCGATACGTATTGTGAATTGCAGATTTTCGTGAAT
CATCGAATCTTTGAACGCACATTGCACCCTCTGGTATTC
CAGAGGGTATGCCTGTTTGAGCGTCATTTCTCTCTCAAA
CCTTCGGGTTTGGTATTGAGTGATACTCTGTCAAGGGTT
15 AACTTGAAATATTGACTTAGCAAGAGTGTACTAATAAG
CAGTCTTTCTGAAATAATGTATTAGGTTCTTCCAACCTCG
TTATATCAGCTAGGCAGGTTTAGAAGTATTTTAGGCTCG
GCTTAACAACAATAAACTAAAAGT 3'

20 SEQ ID NO: 11 DNA sequence for the internal transcribed spacer of
Pichia kluyveri.

5' CTGTGATTTATATCTTATACACATGCGTGAGCGCACCAA
ACACCTAAAATTGTAATAATACCAGTCACTAAGTTTTAA
CAAAACAAAACCTTCAACAACGGATCTCTTGGTTCTCGC
ATCGATGAAGAGCGCAGcGAAATGCGATACCTAGTGTG
25 AATTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCAC
ATTGCGCCCCATGGTATTCCATGGGGCATGCCTGTCTGA
GCGTCGTTTCCTTCTTGCGCAAGCAGAGTTGAGAACAG
GCTATGCCTTTTTCGAAATGGAACGTCGTGGACGAAGT
GAACTAAACTTTTAGCACGCTTTGGCCGCCGAACCTTTA
30 ACTAAGC 3'

SEQ ID NO: 12 DNA sequence for the internal transcribed spacer of
Candida krusei.

5' CTGTGATTTACTACTACTGCGTGAGCGGAACGAAAA
CAACAACACCTAAAATGTGGAATATAGCATATAGTCGA

CAAGAGAAATCTACGAAAAACAAACAAAACCTTTCAAC
AACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAG
CGAAATGCGATACCTAGTGTGAATTGCAGCCATCGTGA
ATCATCGAGTTCTTGAACGCACATTGCGCCCCCTCGGCAT
5 TCCGGGGGGGCATGCCTGTTTGAGCGTCGTTTCCATCTTG
CGCGTGCGCAGAGTTGGGGGAGCGGAGCGGACGACGTG
TAAAGAGCGTCGGAGCTGCGACTCGCCTGAAAGGGAGC
GAAGCTGGCCGAGCGAACTAGACTTTTTTTCAGGGACG
CTTGCGGCCGAGAGCGAGTGTTGCGAGACAACAAAAA
10 GC 3'

SEQ ID NO: 13 DNA sequence for the ITS1 of *Saccharomyces cerevisiae*
and *Saccharomyces bayanus*.

5' AAGAAATTTAATAATTTTGAAAATGGATTTTTTTGTTTT
GGCAAGAGCATGAGAGCTTTTACTGGGCAAGAAGACAA
15 GAGATGGAGAGTCCACCCGGGCCTGCGCTTAAGTGCGC
GGTCTTGCTAGGCTTGTAAGTTTCTTTCTTGCTATTCCA
AACGGTGAGAGATTCTGTGCTTTTGTTATAGGACAATT
AAAACCGTTTCAATACAACACACTGTGGAGTTTTCATAT
CTTTGCAACTTTTTCTTTGGGCATTTCGAGCAATCGGGGC
20 CCAGAGGTAACAAACACAAACAATTTTATCTATTCTTA
AATTTTTGTCAAAAACAAGAATTTTCGTAAGTGGAAATT
TTAAAATATTAA 3'

SEQ ID NO: 14 DNA sequence for the ITS2 of *Saccharomyces cerevisiae*
and *Saccharomyces bayanus*.

25 5' CCTTCTCAAACATTCTGTTTGGTAGTGAGTGATACTCTT
TGGAGTTAACTTGAAATTGCTGGCCTTTTCATTGGATGT
TTTTTTTTCCAAAGAGAGGTTTCTCTGCGTGCTTGAGGT
ATAATGCAAGTACGGTCGTTTATAGGTTTACCAACTGCG
GCTAATCTTTTTTATACTGAGCGTATTGGAACGTTATC
30 GATAAGAAGAGAGCGTCTAGGCGAACAATGTTCTTAAA
GT 3'

SEQ ID NO: 15 DNA sequence for the ITS1 of *Saccharomyces*
ludwigii.

5' AAGAAAAAACTGTTTATAAACAGACGGTAGACTTTTC
TTGGGGAGAGGTTGTTGATTGCTTTGGCCTGCGCTTAAC
TGC GCGGCTAGTAGTTGATGATTTTGTATTATCCGAGA
CGAAGGAAACGTCTGATTTTAAAAACATTATACACTTTG
5 GAGTACTTTTTTTAATGTATTTCTTCCTTGGACGAGCAA
TTGTTCAAGGGTCAATAAACACAAACAATTTTTTTTTTA
TTTAAATTTAAAAATAATTCAAAATATATCATTTCCTTTA
TTAGGAATATAAAAATTTTA 3'

10 SEQ ID NO: 16 DNA sequence for the ITS2 of *Saccharomyces ludwigii*.

5' CCTTCTCAAAGAGTTTTTTTATTCTTTTGGTTGTGAGTG
ATACTCTTTCCTTTTACAGGGAAGGGGTAACTTGAAAT
TGTTGCCTAGCAAAGAAGAATTTTGATTGAAATTTCTTG
15 TTTATTACTATTAGGTTTATCCCACTAGTGATTATTGA
GAGTTTTTATTACAGAGTCTTTTCACTTGCTATAATACT
ATTCTATAAGT 3'

SEQ ID NO: 17 DNA sequence for the ITS1 of *Dekkera bruxellensis*.

20 5' CAGGATGCTGGGCGCAAGCCCGTGCAGACACGTGGATA
AGCAAGGATAAAAATACATTAAATTTATTTAGTTTAGTC
AAGAAAGAATTTTA 3'

SEQ ID NO: 18 DNA sequence for the ITS2 of *Dekkera bruxellensis*.

25 5' CCTTCTCACTATTTAGTGGTTATGAGATTACACGAGGGT
GTTTTCTTCAAAGGAAAGAGGGGAGAGTGAGGGGATAA
TGATTTAAGGTTTCGGCCGTTCAATTATTTTTTTCTTCTCC
CCCAGTTATCAAGT 3'

SEQ ID NO: 19 DNA sequence for the ITS1 of *Dekkera intermedia*.

30 5' CAGGATGCTGGGCGCAAGCCCGTGCAGACACGTGGATA
AGTAAGGATAAAAATACATTAAATTTATTTAGTTTtagt
CAAGAAAGAATTTTA 3'

SEQ ID NO: 20 DNA sequence for the ITS2 of *Dekkera intermedia*.

5' CCTTCTCACTATTTAGTGGTTATGAGATTACACGAGGGT
GTTTTCTTCAAAGGAAAGAGGGGAGAGTGAGGGGATAA
TGATTTAAGGTTTCGGCCGTTCAATTATTTTTCTTCTCCC
5 CCAGTTATCAAGT 3'

SEQ ID NO: 21 DNA sequence for the ITS1 of *Botrytis cinerea*.

5' CAGAGTTCATGCCCCGAAAGGGTAGACCTCCCACCCTTG
TGTATTATTACTTTGTTGCTTTGGCGAGCTGCTTTCGGG
10 CCTTGTATGCTCGCCAGAGAATACCAAACTCTTTTAT
TAATGTCGTCTGAGTACTATATAATAGTTA 3'

SEQ ID NO: 22 DNA sequence for the ITS2 of *Botrytis cinerea*.

5' CAACCCTCAAGCTTAGCTTGGTATTGAGTCTATGTCAGT
AATGGCAGGCTCTAAAATCAGTGGCGGCGCCGCTGGGT
15 CCTGAACGTAGTAATATCTCTCGTTACAGGTTCTCGGTG
TGCTTCTGCCAAAACCCAAATTTTCTATGG 3'

SEQ ID NO: 23 DNA sequence for the ITS1 of *Penicillium crustosum*.

5' CCGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTG
TTTATTTTACCTTGTTGCTTCGGCGGGCCCGCCTTAAT
20 GGCCGCCGGGGGGCTTACGCCCCGGGCCCCGCGCCCGC
CGAAGACACCCTCGAACTCTGTCTGAAGATTGAAGTCT
GAGTGAAAATATAAATTATTTA 3'

SEQ ID NO: 24 DNA sequence for the ITS2 of *Penicillium crustosum*.

5' CTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCCCC
CGATCTCCGGGGGACGGGCCCCGAAAGGCAGCGGCGGCA
25 CCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCG
CTCTGTAGGCCCGGCCGGCGCTTGCCGATCAACCCAAA
TTTTTATCCAGG 3'

SEQ ID NO: 25 DNA sequence for the ITS1 of *Penicillium expansum*.

5' CCGAGTGAGGGCCCTTTGGGTCCAACCTCCCACCCGTGT
 TTATTTACCTCGTTGCTTCGGCGGGCCCGCCTTAACTGG
 CCGCCGGGGGGCTCACGCCCCGGGCCCCGCGCCCGCCG
 AAGACACCCCCGAACCTCTGCCTGAAGATTGTCGTCTGA
 GTGAAAATATAAATTATTTA 3'

SEQ ID NO: 26 DNA sequence for the ITS2 of *Penicillium crustosum*.

5' CTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCTC
 CGATTCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCAC
 CGCGTCCGGTCTTCGAGCGTATGGGGCTTTGTCACCCGC
 TCTGTAGGCCCGGCCGGCGCTTGCCGATCAACCCAAATT
 TTTATCCAGG 3'

SEQ ID NO: 27 DNA sequence for the ITS1 of *Hanseniaspora guilliermondii*.

5' GATTGAATTATCATTGTTGCTCGAGTTCTAGTTTTAGAT
 CTTTTACAATAATGTGTATCTTTATTGAAGATGTGCGCT
 TAATTGCGCTGCTTTTTTAAAGTGTGCGAGTAGAAGTAA
 TCTTGCTTGAATCTCAGTCAACGTTTACACACATTGGAG
 TTTTTTACTTTAATTAAATTCTTTCTGCTTTGAATCGAA
 AGGTTCAAGGCAAAAAACAAACACAAACAATTTATTT
 TATTATAATTTTTTAAACTAAACCAAAATTCCTAACGGA
 AATTTTAAATAATTTA 3'

SEQ ID NO: 28 DNA sequence for the ITS2 of *Hanseniaspora guilliermondii*.

5' CCTTCTCAAAAGATAATTTTTTATTTTTTGGTTGTGGGC
 GATACTCAGGGTTAGCTTGAAATTGAAGATTGTTTCAAT
 CTTTTTAAATTCAACACTTAGCTTCTTTGGAGACGCTGT
 TCTCGCTGTGATGTATTTATGAATTTATTCGTTTTACTTT
 ACAAGGGAAATGGTAATGTACCTTAGGCAAAGGGTTGC
 TTTAATATTCATCAAGT 3'

30 SEQ ID NO: 29 DNA sequence for the ITS1 of *Debaryomyces carsonii*.

5' CAGTATTCTTTTTGCCAGCGCTTAACTGCGCGGCGAAAT
 AAACCTTACACACAATGTTTTTTGTTATTACAGGAACTT

TTGCTTTGGCTTGTCTCTAGAAATAGAGTTGGGCCAAAG
GTTTAAACTAACTTCAATTTATTTGAACTATTTTTCTTAT
TGAAATGTCACCTTGTGATTAAATTCAAAAAATCTTCA
3'

5 SEQ ID NO: 30 DNA sequence for the ITS2 of *Debaryomyces carsonii*.

5' CTCTCTCAAACCTTAGGGTTTGGTATTGAGTGATACTCT
TAGTCGAACTAGGCGTTTGCTTGAAATGTATCGGCATGA
GTGGTACTAGATTAGTGCTTCAGATTTTCAATGTATTA
GGTTTATCCAACCTCGTTGAATAGTCTGATGGCAAGTGT
10 TAGTAACTATGGCTCGGCCTAACAACAACAACAAGT
3'

SEQ ID NO: 31 DNA sequence for the ITS1 of *Pichia anomala*.

5' TAGTATTCTATTGCCAGCGCTTAATTGCGCGGCGATAAA
CCTTACACACATTGTCTAGTTTTTTTGAACCTTGCTTTGG
15 GTGGTGAGCCTGGCTTACTGCCCAAAGGTCTAAACACA
TTTTTTTAATGTTAAACCTTTAACCAATAGTCATGAAA
ATTTTAAACAAAAATTAAAATCTTCA 3'

SEQ ID NO: 32 DNA sequence for the ITS2 of *Pichia anomala*.

5' CTCTCTCAAACCTTCGGGTTTGGTATTGAGTGATACTCT
20 GTCAAGGGTTAACTTGAAATATTGACTTAGCAAGAGTG
TACTAATAAGCAGTCTTTCTGAAATAATGTATTAGGTTC
TTCCAACCTCGTTATATCAGCTAGGCAGGTTTAGAAGTAT
TTTAGGCTCGGCTTAACAACAATAAACTAAAAGT 3'

SEQ ID NO: 33 DNA sequence for the ITS1 of *Pichia kluyveri*.

5' CTGTGATTTATATCTTATACACATGCGTGAGCGCACCAA
25 ACACCTAAAATTGTAATAATACCAGTCACTAAGTTTTAA
CAAAACA 3'

SEQ ID NO: 34 DNA sequence for the ITS2 of *Pichia kluyveri*.

5' CCTTCTTGCGCAAGCAGAGTTGAGAACAGGCTATGCCTT
TTTCGAAATGGAACGTCGTGGACGAAGTGAACATAACT
TTAGCACGCTTTGGCCGCCGAACCTTTAACTAAGC 3'

SEQ ID NO: 35 DNA sequence for the ITS1 of *Candida krusei*.

5 5' CTGTGATTTACTACTACACTGCGTGAGCGGAACGAAAA
CAACAACACCTAAAATGTGGAATATAGCATATAGTCGA
CAAGAGAAATCTACGAAAAAACAACA 3'

SEQ ID NO: 36 DNA sequence for the ITS2 of *Candida krusei*.

10 5' CCATCTTGCGCGTGCGCAGAGTTGGGGGAGCGGAGCGG
ACGACGTGTAAAGAGCGTCGGAGCTGCGACTCGCCTGA
AAGGGAGCGAAGCTGGCCGAGCGAACTAGACTTTTTTT
CAGGGACGCTTGGCGGCCGAGAGCGAGTGTTGCGAGAC
AACAAAAAGC 3'

SEQ ID NO: 37 Oligonucleotide Sequence SXUITS1a.

15 5' TTTTGGCAAGAGCATGAGAGCTTTTACTGGGCAAGAAG
ACAAGAGATGGAGAGTCCACCC 3'

SEQ ID NO: 38 Oligonucleotide Sequence SXLITS2a.

5' CCGTACTTGCATTATACCTCAAGCACGCAGAGAAACCT
CTCTTTGGAAAAAAAAAACA 3'

20 SEQ ID NO: 39 Oligonucleotide Sequence SXUITS1b.

5' TGGCAAGAGCATGAGAGCTTTTACTGGGCAAGAAGACA
AGAGATGGAGAGTCCACCCGGG 3'

SEQ ID NO: 40 Oligonucleotide Sequence SXLITS2b.

25 5' CGACCGTACTTGCATTATACCTCAAGCACGCAGAGAAA
CCTCTCTTTGGAAAAAAAAAAC 3'

SEQ ID NO: 41 Oligonucleotide Sequence SLITS1a.

5' CTGTTTATAAACAGACGGTAGACTTTTCTTGGGGAGAGG
TTGTTGATTGCTTTGGCCTGCGCTTAAGTGC GCGGCTAG
TAGTTGATGATTTTGTATTATCCGAGACGAA 3'

5 SEQ ID NO: 42 Oligonucleotide Sequence SLITS2a.

5' TAATAAACTCTCAATAATCACTAGTTGGGATAAACC
TAATAGTAATAACAAGAAATT 3'

SEQ ID NO: 43 Oligonucleotide Sequence SLITS1b.

10 5' TTTATAAACAGACGGTAGACTTTTCTTGGGGAGAGGTTG
TTGATTGCTTTGGCCTG 3'

SEQ ID NO: 44 Oligonucleotide Sequence SLITS2b.

5' CTGTAATAAACTCTCAATAATCACTAGTTGGGATAA
ACCTAATAGTAATAACAAGAAAT 3'

SEQ ID NO: 45 Oligonucleotide Sequence BRITS1a

15 5' GGTGAACCTGCGGAAGGATCATTACAGGATGCTGGGCG
CAAGCCCGTGCAGACACGTGGATAAGCAAGGATAAAAA
TACAT 3'

SEQ ID NO: 46 Oligonucleotide Sequence BRITS2a.

20 5' ACTGGGGGAGAAGAAAAAATAATGAACGGCCGAAAC
CTTAAATCATTATCCCTCACTCTCCCTCTTTCTTTGA
AGAAAACACCCTCGTGTAATCTCATAACCACTA 3'

SEQ ID NO: 47 Oligonucleotide Sequence BRITS1b.

5' GGTGAACCTGCGGAAGGATCATTACAGGATGCTGGGCG
CAAGCCCGTGCAGACACG 3'

SEQ ID NO: 48 Oligonucleotide Sequence BRLITS2b.

5' AACTGGGGGAGAAGAAAAATAATGAACGGCCGAAAC
CTTAAATCATTATCCCCTCACTCTCCCCTCTTTCCTTTGA
AGAAAACACCCTCGTGTAATCTCATAACCACTA 3'

5 SEQ ID NO: 49 Oligonucleotide Sequence BCUITS1a.

5' TGGCGAGCTGCTTTCGGGCCTTGTATGCTCGCCAGAGAA
TACCAAACTCTTTTAT 3'

SEQ ID NO: 50 Oligonucleotide Sequence BCLITS2.

10 5' GCGCCGCCACTGATTTTAGAGCCTGCCATTACTGACATA
GACTCAATACCAAGCTAA 3'

SEQ ID NO: 51 Oligonucleotide Sequence BCUITS1b.

5' GCTGCTTTCGGGCCTTGTATGCTCGCCAGAGAATACCAA
AACTCTTTTATTAATGT 3'

SEQ ID NO: 52 Oligonucleotide Sequence PXUITS1a.

15 5' CCCTYTGGGTCCAACCTCCCACCCGTCTTTATTTACCT
TGTGCTTCGGCGGGCCCGCC 3'

SEQ ID NO: 53 Oligonucleotide Sequence PXLITS2a.

5' TCGGCAAGCGCCGGCCGGGCCTACAGAGCGGGTGACAA
AGCCCCATACGCTCGAGGACCGGACGCGGTGC 3'

20 SEQ ID NO: 54 Oligonucleotide Sequence PXUITS1b.

5' CCCTYTGGGTCCAACCTCCCACCCGTCTTTATTTACCTC
GTTGCTTCGGCGGGCCCGC 3'

SEQ ID NO: 55 Oligonucleotide Sequence PXLITS2b.

5' AGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCC
ATACGCTCGAGGACCGG 3'

SEQ ID NO: 56 Oligonucleotide Sequence HGUTS1.

5 5' AATCTTGCTTGAATCTCAGTCAACGTTTACACACATTGG
AGTTTTTTTACTTTAATTTAATTCTTTCTGCTTTGAATCG
AAAGGTTCAAGGCAAAAAC 3'

SEQ ID NO: 57 Oligonucleotide Sequence HGLITS2.

10 5' TAAATACATCACAGCGAGAACAGCGTCTCCAAAGAAGC
TAAGTGTTGAATTAAAAAAGATT 3'

SEQ ID NO: 58 Oligonucleotide Sequence DXLITS2a.

5' CTCATGCCGATACATTTCAAGCAAACGCCTAGTTCGACT
AAGAGTATCACTCAATACCAA 3'

SEQ ID NO: 59 Oligonucleotide Sequence DXLITS2b.

15 5' TAAACACTTGCCATCAGACTATTCAACGAGTTGGATAA
ACCTAATACATTGAAAAATCTGA 3'

SEQ ID NO: 60 Oligonucleotide Sequence PAUTS1.

5' TTGCTTTGGGTGGTGAGCCTGGCTTACTGCCCAAAGGTC
TAAACACATTTTTTTAATGT 3'

20 SEQ ID NO: 61 Oligonucleotide Sequence PALITS2.

5' ATACATTATTCAGAAAGACTGCTTATTAGTACACTCTT
GCTAAGTCAATATTTCAAGTTA 3'

SEQ ID NO: 62 Oligonucleotide Sequence PKLITS2.

5' GCGGCCAAAGCGTGCTAAAAGTTTAGTTCACTTCGTCCA
CGACGTTCCATTTCGAAAAAGG 3'

SEQ ID NO: 63 Oligonucleotide Sequence CKUITS1.

5 5' GCGGAACGAAAACAACAACACCTAAAATGTGGAATATA
GCATATAGTCGACAAGAGAAATC 3'

SEQ ID NO: 64 Oligonucleotide Sequence CKLITS2.

5' TCAGGCGAGTCGCAGCTCCGACGCTCTTTACACGTCGTC
CGCTCCGCTCCCCCAACTCT 3'

10 SEQ ID NO: 65 Oligonucleotide Sequence SXU99.

5' CTTTACTGGGCAAGAAGAC 3'

SEQ ID NO: 66 Oligonucleotide Sequence SXL658.

5' AAGCACGCAGAGAAACC 3'

SEQ ID NO: 67 Oligonucleotide Sequence SXU102.

15 5' TTACTGGGCAAGAAGACAAG 3'

SEQ ID NO: 68 Oligonucleotide Sequence SXL661.

5' CTCAAGCACGCAGAGAA 3'

SEQ ID NO: 69 Oligonucleotide Sequence SLU85.

5' GACTTTTCTTGGGGAGAG 3'

SEQ ID NO: 70 Oligonucleotide Sequence SLL635.

5' TCACTAGTTGGGATAAACCT 3'

SEQ ID NO: 71 Oligonucleotide Sequence SLU88.

5' TTTTCTTGGGGAGAGG 3'

5 SEQ ID NO: 72 Oligonucleotide Sequence SLL636.

5' ATCACTAGTTGGGATAAACC 3'

SEQ ID NO: 73 Oligonucleotide Sequence SLU136.

5' CGGCTAGTAGTTGATGATT 3'

SEQ ID NO: 74 Oligonucleotide Sequence SLL634.

10 5' CACTAGTTGGGATAAACCTA 3'

SEQ ID NO: 75 Oligonucleotide Sequence BRU53A.

5' ATTACAGGATGCTGGGC 3'

SEQ ID NO: 76 Oligonucleotide Sequence BRU53B.

5' ATTACAGGATGCTGGG 3'

15 SEQ ID NO: 77 Oligonucleotide Sequence BRU77.

5' CGTGCAGACACGTGGAT 3'

SEQ ID NO: 78 Oligonucleotide Sequence BRL339.

5' CTTTGAAGAAAACACCCTC 3'

SEQ ID NO: 79 Oligonucleotide Sequence BRL367.

5' AATCATTATCCCCTCACTC 3'

SEQ ID NO: 80 Oligonucleotide Sequence BRL390.

5' TAATGAACGGCCGAAAC 3'

5 SEQ ID NO: 81 Oligonucleotide Sequence BCU136.

5' TTGTATGCTCGCCAGAG 3'

SEQ ID NO: 82 Oligonucleotide Sequence BCL393.

5' GCCTGCCATTACTGACA 3'

SEQ ID NO: 83 Oligonucleotide Sequence BCU142.

10 5' GCTCGCCAGAGAATACC 3'

SEQ ID NO: 84 Oligonucleotide Sequence PXU87.

5' ACCCGTCTTTATTTTACCTT 3'

SEQ ID NO: 85 Oligonucleotide Sequence PXL495.

5' CTACAGAGCGGGTGACA 3'

15 SEQ ID NO: 86 Oligonucleotide Sequence PXU86.

5' CACCCGTGTTTATTTTACCT 3'

SEQ ID NO: 87 Oligonucleotide Sequence PXL482.

5' CAAAGCCCCATACGC 3'

SEQ ID NO: 88 Oligonucleotide Sequence PXL491.

5' AGCGGGTGACAAAGC 3'

SEQ ID NO: 89 Oligonucleotide Sequence HGU193.

5' CAACGTTTACACACATTGG 3'

5 SEQ ID NO: 90 Oligonucleotide Sequence HGU231.

5' AATTCTTTCTGCTTTGAATCG 3'

SEQ ID NO: 91 Oligonucleotide Sequence HGL601.

5' CAGCGTCTCCAAAGAAGCTAA 3'

SEQ ID NO: 92 Oligonucleotide Sequence DXL447.

10 5' GCAAACGCCTAGTTCGACTAA 3'

SEQ ID NO: 93 Oligonucleotide Sequence DXL526.

5' ATTCAACGAGTTGGATAAACC 3'

SEQ ID NO: 94 Oligonucleotide Sequence PAU133.

5' GGCTTACTGCCCAAAGGTC 3'

15 SEQ ID NO: 95 Oligonucleotide Sequence PAL451.

5' TGCTTATTAGTACACTCTTGC 3'

SEQ ID NO: 96 Oligonucleotide Sequence PKL356.

5' GTTTAGTTCACTTCGTCCACG 3'

SEQ ID NO: 97 Oligonucleotide Sequence CKU104.

5' CCTAAAATGTGGAATATAGCA 3'

SEQ ID NO: 98 Oligonucleotide Sequence CKL354.

5' ACGCTCTTTACACGTCGTC 3'

5 SEQ ID NO: 99 Oligonucleotide Sequence ITS5.

5' GGAAGTAAAAGTCGTAACAAGG 3'

SEQ ID NO: 100 Oligonucleotide Sequence ITS4.

5' TCCTCCGCTTATTGATATGC 3'

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04251

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C07H 21/04

US CL :435/6; 536/24.32, 24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/24.32, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| X | US 5,631,132 A (LOTT et al) 20 May 1997, column 4. | 4 |
| X,P | US 5,792,611 A (HAMELIN) 11 August 1998, column 5. | 4 |



Further documents are listed in the continuation of Box C.



See patent family annex.

| | |
|---|--|
| * Special categories of cited documents: | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *A* document defining the general state of the art which is not considered to be of particular relevance | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| *B* earlier document published on or after the international filing date | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *Z* document member of the same patent family |
| *O* document referring to an oral disclosure, use, exhibition or other means | |
| *P* document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

01 JULY 1999

Date of mailing of the international search report

20 JUL 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
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PARALEGAL SPECIALIST
CHEMICAL MATRIX

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04251

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-3 and 5-45
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claims are unsearchable to the extent that they reference specific nucleotide sequences from the Sequence Listing. Because a computer readable copy of the Sequence Listing has not been submitted, a meaningful search cannot be carried out by this Authority.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04251

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS; WEST; DIALOG: medline, CA, biosis, embase, scisearch

search terms: ITS1, ITS2, internal transcribed spacer, intergenic spacer, *Saccharomyces cerevisiae* or *bayanus*,
Saccharomyces ludwigii, *Dekkera bruxellensis* or *intermedia*, *Botrytis cinerea*, *Penicillium crustosum* or *expansum*,
Hanseniaspora guilliermondii, *Debaryomyces carsonii*, *Pichia anomala* or *kluveri* or *Candida krusei*

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Summary

| Document | Pages | Printed | Missed |
|--------------------|--------------|----------------|---------------|
| WO009946405 | 53 | 53 | 0 |
| Total (1) | 53 | 53 | 0 |